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Toll-like Receptor 4 Mediates Induction of the Bcl10-NFκB-Interleukin-8 Inflammatory Pathway by Carrageenan in Human Intestinal Epithelial Cells*

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Sumit Bhattacharyya[‡], Ravinder Gill[‡], Mei Ling Chen[§], Fuming Zhang[¶], Robert J. Linhardt[¶], Pradeep K. Dudeja[‡‖], and Joanne K. Tobacman[‡‖1]

From the [‡]Department of Medicine and [§]Department of Anatomy and Cell Biology, University of Illinois, Chicago, Illinois 60612, [‖]Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois 60612, and [¶]Department of Chemistry and Chemical Biology, Rensselaer Polytechnic Institute, Troy, New York 12180

The sulfated polysaccharide carrageenan (CGN) induces activation of NFκB and interleukin 8 (IL-8) in human colonic epithelial cells through a pathway of innate immunity mediated by Bcl10 (B-cell CLL/lymphoma 10). In this report, we identify Toll-like receptor 4 (TLR4), a member of the family of innate immune receptors, as the surface membrane receptor for CGN in human colonic epithelial cells. Experiments with fluorescence-tagged CGN demonstrated a marked reduction in binding of CGN to human intestinal epithelial cells and to RAW 264.7 mouse macrophages, following exposure to TLR4 blocking antibody (HTA-125). Binding of CGN to 10ScNCr/23 mouse macrophages, which are deficient in the genetic locus for TLR4, was absent. Additional experiments with TLR4 blocking antibody and TLR4 small interfering RNAs showed 80% reductions in CGN-induced increases in Bcl10 and IL-8. Transfection with dominant-negative MyD88 plasmid demonstrated MyD88 dependence of the CGN-TLR4-triggered increases in Bcl10 and IL-8. Therefore, these results indicate that CGN-induced inflammation in human colonocytes proceeds through a pathway of innate immunity, perhaps related to the unusual α-1,3-galactosidic linkage characteristic of CGN, and suggest how dietary CGN intake may contribute to human intestinal inflammation. Because CGN is a commonly used food additive in the Western diet, clarification of its effects and mechanisms of action are vital to issues of food safety.

Toll-like receptor 4 (TLR4)[2] is a member of the family of innate immunity receptors that are activated by a wide variety of microbial products, including lipopolysaccharide (LPS), lipoteichoic acid, and peptidoglycan (1–3). Recently, we found that the sulfated polygalactose carrageenan (CGN), composed of sulfated galactose residues in alternating α-1,3 and β-1,4 galactosidic linkages, induces activation of NFκB and IL-8 in human intestinal epithelial cells (IEC) through a pathway of innate immunity mediated by Bcl10 (4). CGN, in λ-, κ-, and ι-forms, is widely used as a food additive in the Western diet, so its induction of an inflammatory cascade in the human intestine may have profound implications for human disease. CGN has been used widely for decades to induce intestinal inflammation and neoplasms in animal models of inflammatory bowel disease, yet it is only recently that the Joint FAO/WHO Expert Committee on Food Additives (JECFA) judged that it is inadvisable to include CGN in human food, specifically infant formula, and recommended a new dietary exposure evaluation be undertaken for CGN (5). Because CGN is incorporated widely into processed foods in the diet, we are interested in determining its effects on the human intestinal epithelium and the precise mechanisms involved.

The chemical structure of CGN consists of sulfated galactose residues that are connected in α-1,3 and β-1,4 linkages. The sites and extent of sulfations and secondary branching may vary among the λ, κ, and ι types of CGN, which are all derived from red seaweed. All CGN contains the unusual α-1,3-galactosidic linkage, which is recognized in humans by the anti-Gal antibody (6, 7). The α-1,3-galactosyltransferase gene, which is required to form α-1,3-galactosyl linkages, is nonfunctioning in the human and Old World ape genome, leading to absence of the α-1,3-galactosyl bonds. In contrast, other mammals and many primates have this gene and are able to form this galactosidic linkage (6). Rejection of transplanted nonhuman tissues, such as pig skin, is related to the activation of this immune response (8, 9).

To elucidate the mechanism of the effects of CGN on inflammation, we have investigated the interaction between CGN and the Toll-like receptor 4 (TLR4), because TLR4 is known to interact with Bcl10 in an inflammatory cascade in immune cells (10). Previously, we demonstrated that CGN exposure induces a pathway of activation of NFκB and IL-8 in human intestinal epithelial cells (IEC); this pathway is mediated by Bcl10 (B-cell CLL/lymphoma 10) (4). Bcl10, recognized as a mediator of innate inflammation, is up-regulated following translocations

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[1] To whom correspondence should be addressed: University of Illinois at Chicago, Dept. of Medicine, 840 S. Wood St., M/C 718, 440 Clinical Sciences North, Chicago, IL 60612. Tel.: 312-569-7826; Fax: 312-413-8283; E-mail: jkt@uic.edu.

[2] The abbreviations used are: TLR, Toll-like receptor; LPS, lipopolysaccharide; CGN, carrageenan; CGN-F, fluorescent CGN; CGN-E, excess unlabeled λCGN; IL, interleukin; IEC, intestinal epithelial cells; PMB, polymyxin B sulfate; PMBN, polymyxin B nonapeptide; ELISA, enzyme-linked immunosorbent assay; QRT, quantitative real-time; CARD, caspase recruitment domain; LRR, leucine-rich repeat; siRNA, small interfering RNA; IRAK, receptor-associated kinase; FITC, fluorescein isothiocyanate; ANOVA, analysis of variance.

in MALT (mucosa-associated lymphoid tissue) lymphomas and is associated with constitutive activation of NFκB (11–13).

Identification of a membrane receptor for CGN in IEC facilitates elucidation of how CGN initiates an inflammatory response. When TLR4 reacts with lipopolysaccharide, an inflammatory cascade is instigated that culminates in production of chemokines, including IL-8 and IL-6, and an inflammatory infiltrate with macrophages, lymphocytes, and polymorphonuclear leukocytes (14, 15). The Toll family of membrane receptors are pattern recognition receptors that sense conserved motifs, predominantly the pathogen-associated molecular patterns (16). When LPS interacts with TLR4, the inflammatory cascade that is activated can lead to systemic inflammatory response syndrome and sepsis in the clinical setting (17). Specific modifications of TLR4 have been associated with altered endotoxin responsiveness (18, 19).

The interaction that we demonstrate in this report between CGN and TLR4 may help to explain how exposure to CGN induces an inflammatory cascade associated with increases in Bcl10, phospho-IκBα, nuclear NFκB, and IL-8 secretion in human epithelial cells. Because TLR4 is increasingly recognized as a mediator of inflammation in metabolic disorders associated with insulin resistance, the association between CGN and the TLR4 may be relevant to the induction of other disorders as well as to inflammatory bowel disease (20, 21).

EXPERIMENTAL PROCEDURES

NCM460 Cell Culture—NCM460 is a human colonic epithelial cell line, originally derived from the normal colon mucosa of a 68-year-old Hispanic male (22). NCM460 cells were grown in M3:10™ medium (INCELL Corp., San Antonio, TX) and maintained at 37 °C in a humidified 5% CO_2 environment with media renewal at 2-day intervals. Confluent cells in T-25 flasks (Costar) were harvested by trypsinization and subcultured in multiwell tissue culture clusters (Costar). Cells were treated with lambda-carrageenan (λCGN; 1 μg/ml, Sigma-Aldrich) for 24 h under the conditions indicated above unless stated otherwise. Spent media were collected from the control and treated wells at specified times and stored at −80 °C. Cells were harvested by scraping, and total cell protein was measured using the BCA™ protein assay kit (Pierce) with bovine serum albumin as the standard.

Human Colonic Tissue Specimens and Primary Cell Culture—De-identified colon specimens were obtained at the time of colectomy through an established protocol approved by the Institutional Review Board of the University of Illinois at Chicago (UIC), which enables access to surgical specimens by the Tissue Bank of the UIC Hospital and the Department of Pathology. Patients consented to donate tissue to the tissue bank for research purposes. Normal tissue that was several centimeters away from any macroscopic lesion was isolated and maintained under sterile conditions, and small tissue samples were obtained by scraping from the underlying muscle. Primary colonocyte cultures were prepared as described previously (4). Briefly, colonocytes were dispersed from the scraped tissue by digestion with collagenase IV (Worthington Biochemical Corp.) for 6 h. Dispersed cells were collected after centrifugation, cultured in M3:10™ media, and maintained at 37 °C in a humidified, 5% CO_2 environment. In addition, some fragments of scraped tissue were placed in 12-well cell cluster dishes in Dulbecco's modified Eagle's medium with Pen-Strep (Sigma) antibiotics and 10% fetal bovine serum as described previously (4). Half of the samples were exposed to λCGN (1–10 μg/ml for 1–2 h). Treated and control samples were deep frozen, and subsequently 5-μm tissue sections were cut by microtome and slides were prepared. Control and treated tissue sections on slides were processed simultaneously and stained for immunohistochemistry or confocal microscopy.

Mouse Macrophage Cell Lines—Mouse macrophage cell lines obtained from ATCC were grown under the recommended conditions. The RAW 264.7 cell line (ATCC TIB-71) is a normal mouse macrophage cell line that expresses TLR4 (23). Cells were grown in Dulbecco's modified Eagle's medium with 4 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, and 10% fetal bovine serum at 37 °C, 5% CO_2 in 96-well plates. The mouse macrophage cell line 23ScCr (10 ScNCr/23; ATCC CRL-2751), which is deficient in the genetic locus for TLR4, was grown under similar conditions (24).

Fluorescence-tagged λ-Carrageenan—Undegraded λCGN (Sigma) of $M_r \sim 1 \times 10^6$ was tagged with fluoresceinamine (Sigma) following activation of λCGN hydroxyl groups with CNBr, pH 11, using a modification of a standard protocol (25). The fluorescently labeled λCGN was purified using ultrafiltration (molecular weight cutoff, 10,000) and Sephadex G25 chromatography to obtain labeled λCGN free of fluoresceinamine. It was tagged on less than 1% of its hydroxyl groups and detected in a microplate fluorescence reader (FL600, Bio-Tek Instruments, Inc., Winooski, VT) with 485 nm excitation and 540 nm emission filters.

Polymyxin B Inhibits LPS-, but Not CGN-, Induced Increase in IL-8—NCM460 cells were exposed to CGN (1 μg/ml) and LPS (10 ng/ml, Sigma; from *Escherichia coli* 026:B6) for 6 h in the presence and absence of polymyxin B sulfate (PMB) (10 μg/ml; Sigma) to determine whether PMB had an inhibitory effect on the secretion of IL-8, because PMB is a known inhibitor of LPS (26, 27). Subsequently, IL-8 was measured by enzyme-linked immunosorbent assay (ELISA) as described below. A derivative of PMB, polymyxin B nonapeptide hydrochloride (PMBN; Sigma; 60 ng/ml), was also tested for its impact on IL-8 secretion in response to LPS and CGN.

ELISA for IL-8—The ELISA method for detection of IL-8 was reported previously by our laboratory (4). The secretion of IL-8 in the spent media of control and treated NCM460 cells and primary colon cells was measured by the DuoSet ELISA kit for human IL-8 (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. The range of detection of the standard curve is 31.2–2000 pg/ml with a sensitivity of 1.5–7.5 pg/ml. The IL-8 in the spent media was captured into the wells of a microtiter plate precoated with specific anti-IL-8 monoclonal antibody. Immobilized IL-8 was then detected by biotin-conjugated secondary IL-8 antibody and streptavidin-horseradish peroxidase. Hydrogen peroxide/tetramethyl-benzidine chromogenic substrate was used to develop the color and intensity of color was measured at 450 nm with a reference filter of 570 nm in an ELISA plate reader (Spectra II, Tecan). The IL-8 concentrations were extrapolated from a standard curve plot-

ted using known concentrations of IL-8. The sample values were normalized by the total protein content (BCA™ protein assay kit, Pierce) and expressed as pg or ng/mg cellular protein.

ELISA for Bcl10—The expression of Bcl10 in NCM460 cells or primary colon cells was determined by a solid-phase sandwich ELISA designed to quantify cellular Bcl10 (28). Control or treated cells were lysed in radioimmune precipitation assay buffer (50 mM Tris-HCl containing 0.15 M NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1% SDS, pH 7.4), and cell extracts were stored at −80 °C until assayed. Bcl10 molecules in the samples or standards were captured in the wells of a microtiter plate precoated with rabbit polyclonal antibody to Bcl10 (QED Bioscience Inc., San Diego, CA). Immobilized Bcl10 molecules were detected by a mouse monoclonal antibody to Bcl10 (Novus Biologicals, Littleton, CO) and goat anti-mouse IgG-horseradish peroxidase complex (Santa Cruz Biotechnology, Santa Cruz, CA). The peroxidase enzyme activity bound to Bcl10 molecules was determined by chromogenic reaction with hydrogen peroxide/tetramethylbenzidine. Color development due to enzymatic activity was stopped by 2 N sulfuric acid, and intensity of color was measured at 450 nm in an ELISA plate reader (SLT, Spectra). Bcl10 concentrations of the samples were extrapolated from a standard curve derived using known concentrations of recombinant Bcl10 (Calbiochem, EMD Biosciences, Carlsbad, CA). Sample values were normalized by the total cell protein concentrations determined by the BCA™ protein assay kit (Pierce).

TLR4 Inhibition by Blocking Antibody—NCM460 cells were grown in 12-well tissue culture plates. At 70–80% confluency, the cells were treated for 2 h with fresh media containing 10 or 20 μg/ml TLR4 receptor-blocking antibody (HTA-125; BioLegend) or IgG2α prior to λCGN (1 and 10 μg/ml) exposure for 24 h unless otherwise specified. Cells were incubated at 37 °C in a humidified 5% CO_2 environment. After 24 h, the spent medium was collected for IL-8 measurement, and the cells were harvested for Bcl10 determination by ELISA.

TLR4 Knockdown by Small Interfering RNA (siRNA)—siRNA for TLR4 was obtained commercially (Qiagen Inc., Valencia, CA), and two were found to effectively reduce TLR4 activity in NCM460 cells. 150 ng (0.6 μl) of TLR4 siRNA in 100 μl of serum-free culture medium was mixed with 12 μl of HiPerfect transfection reagent (Qiagen) by vortexing, maintained at room temperature for 10 min, and then added dropwise onto the NCM460 cells in a 6-well culture plate. Cells were swirled for uniform distribution of the transfection complex, and the transfected cells were incubated at 37 °C and 5% CO_2. Subsequently, 24 h later cells were exposed to λCGN at a concentration of 1 μg/ml for an additional 24 h. Media were collected, and IL-8 secretion in the spent media and Bcl10 content of the cell lysate were measured by ELISA.

Detection of CGN Binding by Measurement of Fluorescent CGN—NCM460 cells, normal human colonocytes, mouse macrophage cell line RAW 264.7, and mouse macrophage cell line 23ScCr, which is deficient in TLR4, were grown in 96-well tissue culture plates (23, 24). Cells were 70–80% confluent when treated with fluorescence-tagged λCGN (1 μg/ml for 2 h) following incubation with TLR4 blocking antibody (HTA-125, BioLegend; 10 μg/ml) for 2 h. In competitive binding experiments, 70–80% confluent cells were exposed to fluorescence-tagged λCGN (1 μg/ml for 2 h) following a 2-h preincubation with (*a*) isotype IgG2α control (10 μg/ml) or (*b*) TLR4 blocking antibody (HTA-125, Biolegend; 10 μg/ml). Excess unlabeled λCGN (5 μg/ml) was added at the same time as the fluorescent λCGN (1 μg/ml). Cells were incubated at 37 °C in 5% CO_2. Controls also included FITC-tagged IgG2α (10 μg/ml) for 2 h. At the termination of the incubation, the cells were washed thoroughly to remove unbound fluorescence-tagged λCGN. 100 μl of Tris-Cl buffer, pH 7.4, was added to each well, and fluorescence was detected using a microplate fluorescence reader (FL600, Bio-Tek Instruments) at 485 nm excitation wavelength with a 540 nm emission filter. The effect of increasing concentration of TLR4 blocking antibody (0.1, 1, and 10 μg/ml for 2 h) on the binding of fluorescent CGN to the NCM460 cells was also determined by this approach.

QRT-PCR of TLR4—To determine if there were any change in the expression of TLR4 following CGN exposure, real-time semiquantitative polymerase chain reaction was performed using the Mx3000P® (Stratagene, La Jolla, CA). Total RNA was extracted and purified from cultured NCM460 cells by an RNeasy mini-kit (Qiagen). Equal amounts of purified RNAs from the control and treated cells were reverse-transcribed and amplified using Brilliant® SYBR Green QRT-PCR Master Mix (Stratagene). Human β-actin was used as an internal control. Cycle threshold (Ct) was determined for TLR4 and β-actin expression during the exponential phase of amplification. Primers for TLR4 (NCBI NM_138554) were designed using the Primer3 program[3]: Forward: 5-GGATGAGGACTGGGTAA-3′; Reverse: 5-TGGGACACCACAACAATCAC-3′. Fold changes in expression were determined from the differences between the Ct values using the formulae: Fold change = 2^{Δ_3} with $\Delta_3 = \Delta_1 - \Delta_2$; $\Delta_1 = Ct_{\text{control target gene}} - Ct_{\text{control actin}}$; and $\Delta_2 = Ct_{\text{treated target gene}} - Ct_{\text{treated actin}}$.

Inhibition of Interleukin 1b Receptor-associated Kinase (IRAK) by Selective Inhibitor—IRAK1/4 was inhibited by exposure of the NCM460 cells to the selective inhibitor *N*-(2-morpholinylethyl)-2-(3-nitrobenzoyl-amido)-benzimidazole (EMD Biosciences) at a concentration of 50 μM for 2 h (30). Concentrations of IL-8 in the spent media and Bcl10 in the cell lysate were measured following this pretreatment and exposure to 1 μg/ml λCGN for 24 h at 37 °C and 5% CO_2.

Transfection with Dominant-Negative MyD88 Plasmid—MyD88 is recognized as an adaptor molecule for the TLR4-induced activation of an inflammatory cascade in immune cells and pulmonary epithelial cells (31, 32). To determine the involvement of MyD88 in the CGN-TLR4 interaction and induction of inflammation in the IEC, short hairpin RNA designed to silence MyD88 (NCBI NM_002468) was introduced into the NCM460 cells (InvivoGen, San Diego, CA). The siRNA was obtained in a plasmid downstream of the RNA polymerase III promoter (7SK). Lyophilized plasmid was centrifuged to pellet the DNA, and DNA was resuspended and amplified in *E. coli* GT116 using Fast-Media® Zeo (InvivoGen). Vector control psiRNA-LucGL3 was processed in parallel to

[3] Contact the author for further information.

TABLE 1
Effects of polymyxin B on LPS- and CGN-induced increases in IL-8

LPS	CGN	PMB	PMBN	IL-8
ng/ml	*μg/ml*	*μg/ml*	*μg/ml*	*pg/ml ± S.D.*
0	0	0	0	160 ± 15
10	0	0	0	2651 ± 56
10	0	10	0	164 ± 8
10	0	0	60	183 ± 1
0	1	0	0	325 ± 24
0	1	10	0	303 ± 20
0	1	0	60	319 ± 32
0	0	10	0	159 ± 13
0	0	0	60	161 ± 14
10	1	0	0	2854 ± 126

the psiRNA-hMyD88 plasmid DNA, and NCM460 cells were transfected using the HiPerfect transfection reagent (Qiagen). 24 h post-transfection, cells were treated with λCGN (1 μg/ml x 24 h), and measurement of Bcl10 in the cell lysate and IL-8 in the spent media was performed by ELISA.

Measurement of KC in Mouse Macrophage Cells and Mouse Colonic Tissue—Mouse macrophages (RAW 264.7 and 23ScCr) were grown in Dulbecco's modified Eagle's medium with 10% FBS and exposed to λCGN (1 μg/ml for 24 h). Spent media were collected and assayed for KC, the mouse equivalent of human IL-8, by ELISA (R&D Systems).

Mice with a deletion of the TLR4 gene (C57BL/10ScNJ) were obtained from The Jackson Laboratory (Bar Harbor, ME), as well as normal, age-matched controls (C57BL/10ScSnJ). Mice were euthanized at 9 weeks and the colon dissected. Small fragments of colon were placed in wells of a 24-well tissue culture plate, exposed to λCGN (1 μg/ml), and incubated at 37 °C and 5%CO_2. At 2 and 6 h, spent media were collected from six samples and tested for KC by ELISA.

Confocal Microscopy of Human Intestinal Cells Probed for TLR4—NCM460 cells and primary human colonic epithelial cells were grown on collagen-coated Transwell inserts or four-chamber tissue culture slides for 24 h, and then treated preparations were exposed to 1 μg/ml CGN for 24 h. Methods for staining and examining cells by confocal microscopy were described previously (33). Cells were washed once in 1× phosphate-buffered saline containing 1 mM calcium chloride (pH 7.4), fixed for 1.5 h with 2% paraformaldehyde, and then permeabilized with 0.08% saponin. Preparations were washed with phosphate-buffered saline, blocked in 5% normal goat serum, incubated overnight with TLR4 polyclonal antibody mapping to an internal epitope (SC-10741; 1:100, Santa Cruz) at 4 °C, and then washed and stained with goat anti-rabbit Alexa Fluor IgG 568 (1:100, Invitrogen). Cells were exposed for 1 h to phalloidin-Alexa Fluor 488 (Invitrogen) diluted 1:40 to stain actin and to Hoechst 33342 (1:20,000, Invitrogen) for nuclear staining. Preparations were washed thoroughly, mounted, and observed using a Zeiss LSM 510 laser-scanning confocal microscope equipped with a ×63 water immersion objective. Excitation was at 488 and 534 nm from an argon/krypton laser and at 361 nm from a UV laser. Green and red fluorescence were detected through LP505 and 585 filters. The fluorochromes were scanned sequentially, and the collected images were exported with Zeiss LSM Image Browser software as TIFF files for analysis and reproduction.

Statistical Analysis—Statistical analysis was performed using GraphPad InStat software (GraphPad Software, San Diego, CA). One-way ANOVA tests with a Tukey-Kramer multiple comparison test for post-test correction were used for all statistical tests unless a different statistical test is indicated. A two-tailed, paired *t* test was used to compare the results of the QRT-PCR measurements. A *p* value of <0.05 was considered statistically significant. Measurements are the mean ± S.D. of at least three independent biological determinations with technical replicates of each measurement; *** represents $p < 0.001$, unless stated otherwise. Values are expressed as ±S.D.

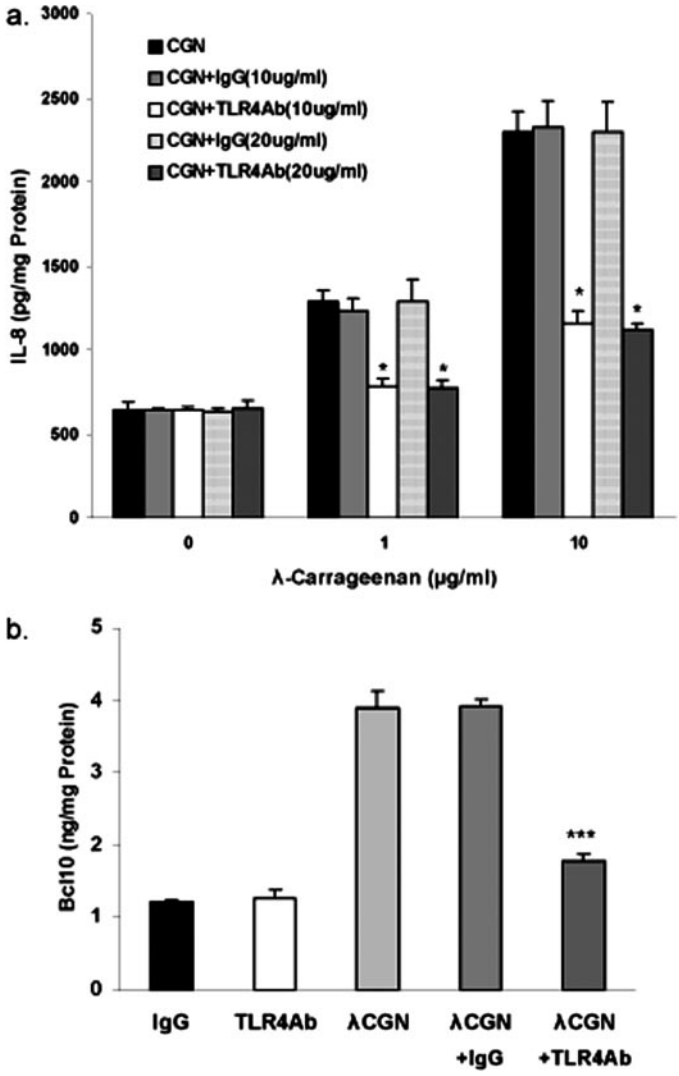

FIGURE 1. **Effects of blocking antibody for TLR4 on CGN-induced increases in IL-8 and Bcl10.** *a*, IL-8 was measured following exposure of NCM460 cells to λCGN (1 and 10 μg/ml for 24 h) following pretreatment with TLR4 blocking antibody (HTA-125) at concentrations of 10 and 20 μg/ml for 2 h. A dose-response effect is seen, with a reduced concentration of IL-8 in relation to a higher concentration of TLR4 blocking antibody. IL-8 in spent media was measured by ELISA and is expressed in pg/mg cell protein. Declines in IL-8 are statistically significant (*, $p < 0.001$). *b*, Bcl10 in the cell lysate was measured under conditions similar to those in *A*, with λCGN (1 μg/ml) and TLR4 antibody (10 μg/ml). An 80% decline in the Bcl10 protein occurred (***, $p < 0.001$).

RESULTS

Effect of the LPS Inhibitor Polymyxin B—To exclude contamination of λCGN by LPS, a known activator of IL-8 following inter-

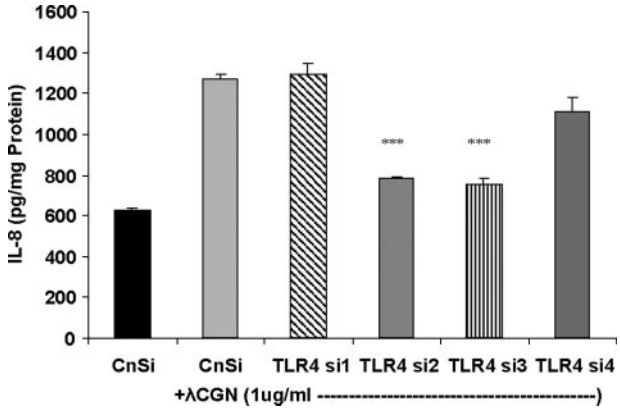

FIGURE 2. **Effect of silencing TLR4 on IL-8 production in NCM460 cells.** When NCM460 cells were treated with siRNA for TLR4 for 24 h prior to exposure to λCGN (1 μg/ml for 24 h), a marked decline in production of IL-8 occurred (***, $p < 0.001$). *CnSi*, control siRNA.

action with TLR4, we tested the effect of the LPS inhibitor polymyxin B sulfate (10 μg/ml), and the PMB derivative, polymyxin B nonapeptide (60 μg/ml) on CGN-induced IL-8 production (Table 1). Prior treatment with PMB or PMBN abrogated the LPS (10 ng/ml for 6 h)-induced increase in IL-8. In contrast, PMB or PMBN had no effect on the CGN (1 μg/ml for 6 h)-induced increase in IL-8, which remained at 200% of base line.

Effect of TLR4 Blocking on IL-8 and Bcl10—Pretreatment with TLR4 blocking antibody (HTA-125) significantly reduced the CGN-induced increases in IL-8 (Fig. 1*a*) and Bcl10 (Fig. 1*b*) ($p < 0.001$). NCM460 cells were pretreated with TLR4 blocking antibody (10 or 20 μg/ml for 2 h) followed by λCGN (1 or 10 μg/ml) for 24 h. λCGN (10 μg/ml) induced an increase in IL-8 from the base-line value of 649 ± 38 to 2296 ± 129 pg/ml. In the presence of TLR4 blocking antibody (10 and 20 μg/ml), IL-8 declined to 1163 ± 72 and 1122 ± 33 pg/ml, respectively. Following TLR4 blocking antibody, the λCGN-induced increases in IL-8 declined by 78 and 81%, respectively. No effect of IgG2α control was evident. Declines were statistically significant ($p < 0.001$).

Similarly, increase in Bcl10 diminished from 3.90 ± 0.23 to 1.79 ± 0.09 ng/ml following TLR4 blocking antibody (10 μg/ml for 2 h) prior to CGN exposure (1 μg/ml for 24 h). Isotype IgG2α control had no effect on Bcl10 or IL-8 levels. Incubation with TLR4 blocking antibody alone produced no decline in the IL-8 production in the NCM460 cells. TLR4 blocking antibody reduced by 78% the CGN-induced increase in Bcl10.

TLR4 siRNA Reduces CGN-induced IL-8 Increase—Silencing of TLR4 by siRNA produced results similar to those obtained with TLR4 blocking antibody (Fig. 2). Significant decline in IL-8 occurred following silencing, with IL-8 declining from 1296 ± 51 to 756 ± 28 pg/ml after exposure to CGN (1 μg/ml for 24 h), a decline of more than 80%. The base-line value was 627 ± 7 pg/ml.

Increased TLR4 mRNA following CGN Exposure—QRT-PCR demonstrated an increase in mRNA for TLR4 following exposure to λCGN in the NCM460 cells. TLR4 mRNA following CGN exposure was 1.65 ± 0.18 times the control. This difference is statistically significant ($p = 0.02$, paired *t* test, two-tailed).

Inhibition of Binding of Fluorescent CGN—NCM460 cells were preincubated for 2 h with increasing concentrations of TLR4 blocking antibody (0.1, 1 and 10 μg/ml). The binding of

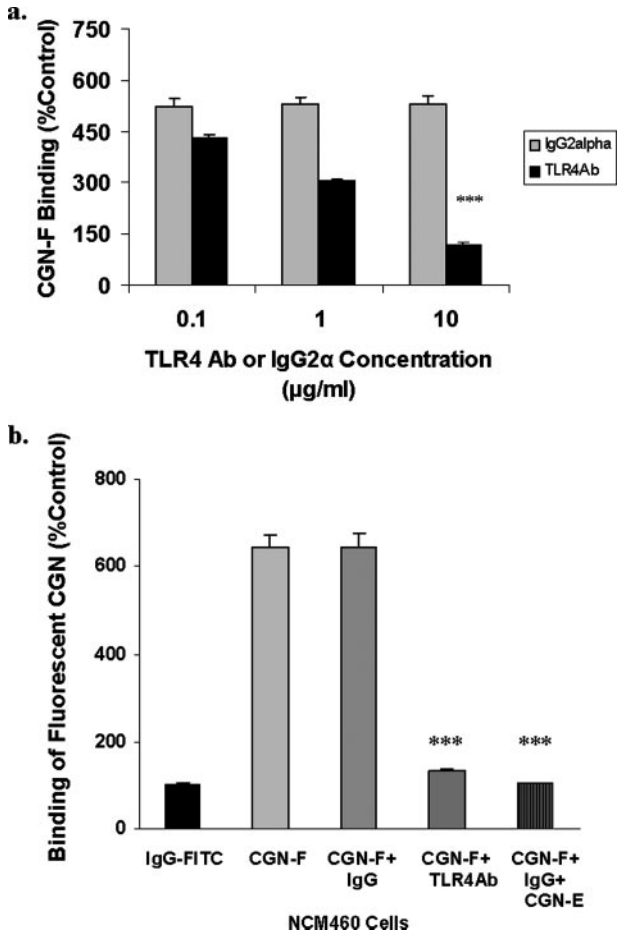

FIGURE 3. **Reduced binding of fluorescent carrageenan with increasing concentration of TLR4 blocking antibody and excess, unlabeled CGN in NCM460 cells.** *a*, in the NCM460 cells, pretreatment with TLR4 blocking antibody (*TLR4 Ab*) (0.1, 1, and 10 μg/ml for 2 h) reduced the uptake of CGN-F (1 μg/ml for 2 h) in a dose-dependent manner from 83 to 23% of control, in contrast to the IgG2α control (***, $p < 0.001$, one-way ANOVA with Tukey-Kramer post-test; $n = 6$, with technical replicates of each determination). *b*, competition with CGN-E (5 μg/ml), which was added simultaneously with labeled CGN (1 μg/ml for 2 h), produced a marked decline in the uptake of fluorescent CGN (***, $p < 0.001$). IgG-FITC was used as internal control for binding of nonspecific fluorescence.

fluorescent CGN (CGN-F) to the cells declined progressively from 83 to 57 to 23% of the control level (Fig. 3*a*). Control isotype IgG had no effect on the binding of CGN-F. Similarly, excess unlabeled λCGN (CGN-E; 5 μg/ml) completely inhibited the attachment of CGN-F to the NCM460 cells (Fig. 3*b*). Results are statistically significant ($p < 0.001$).

In the mouse macrophage cell line 23ScCr, which has the genetic defect of the TLR4 locus and was tested as a negative control, no binding of CGN-F occurred (Fig. 4*a*). Exposure to TLR4 binding antibody or CGN-E had no effect on the binding of CGN-F. In the mouse macrophage cell line RAW 264.7 (ATCC TIB-71), binding of CGN-F was inhibited following pretreatment for 2 h with TLR4 blocking antibody (10 μg/ml) or in the presence of CGN-E (Fig. 4*b*). In primary normal human colonocytes, the effects of TLR4 blocking antibody and of CGN-E on the binding of CGN-F were similar to the results in the NCM460 and RAW 264.7 cells (Fig. 4*c*). Isotype IgG2α control for the TLR4 antibody had no effect on the uptake of CGN-F. Nonspecific fluorescence of IgG-FITC control was

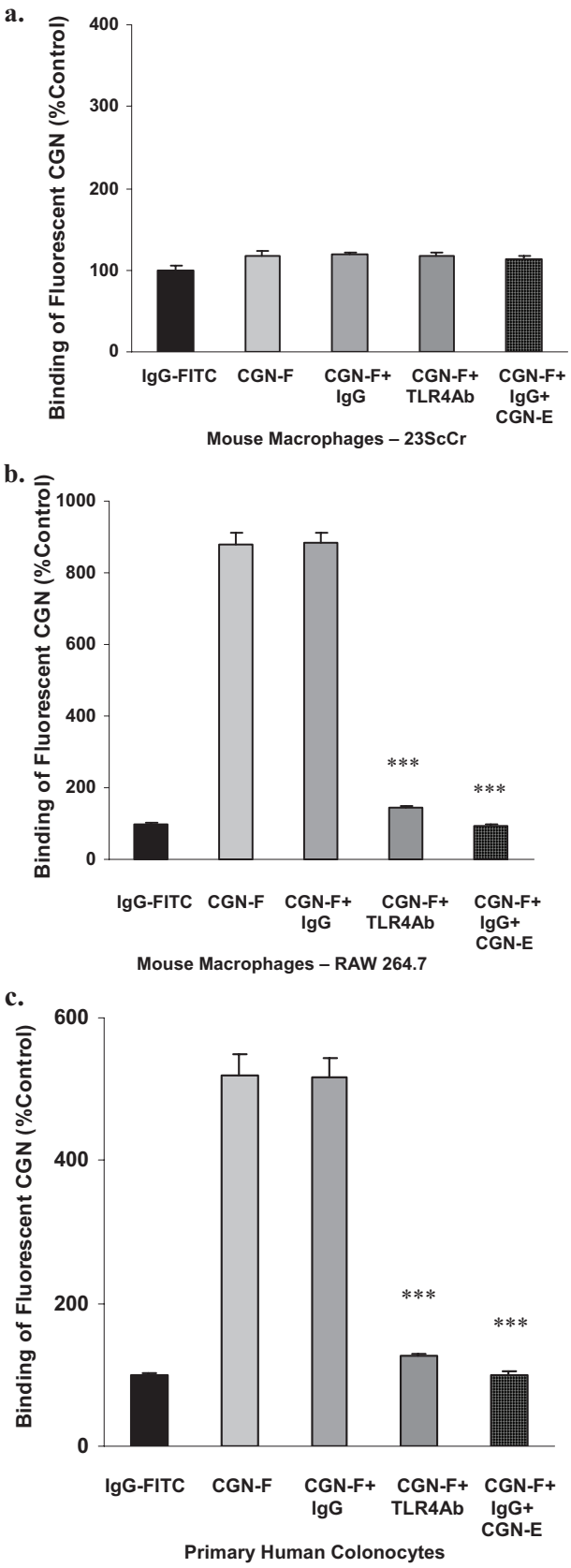

FIGURE 4. **Binding of fluorescent carrageenan in human colonocytes and in mouse macrophage cell lines in the presence of TLR4 blocking antibody and excess unlabeled CGN.** *a*, in contrast to the NCM460 cells, in the mouse 23ScCr cell line, no increase in uptake of fluorescent CGN occurred, consistent with the absence of the genetic locus for TLR4 in these cells. IgG2α was used as the IgG isotype control, and IgG-FITC was used as the internal control for uptake of nonspecific fluorescence. Results were obtained from six experiments, with technical duplicates of each measurement. *b*, in the RAW 264.7 mouse macrophage cell line, which has receptors for TLR4, a significant decline in uptake of fluorescent CGN (1 μg/ml for 2 h) occurred following preincubation with the TLR4 blocking antibody (10 μg/ml for 2 h) or combined exposure with CGN-E (5 μg/ml). *c*, in primary human colonocytes, similar results were obtained with marked reduction in uptake of CGN-F by the human colonocytes following preincubation with the TLR4 blocking antibody or in the presence of CGN-E. ***, $p < 0.001$.

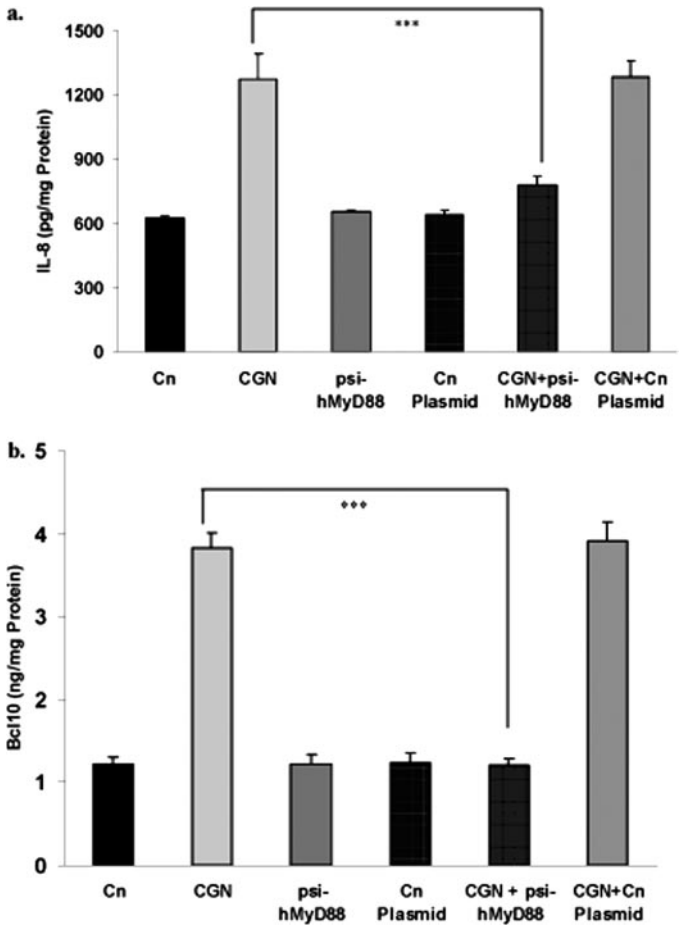

FIGURE 5. **CGN-induced increase in Bcl10 and IL-8 is MyD88-dependent.** *a*, MyD88 was silenced by insertion of a dominant-negative plasmid for MyD88. CGN-induced IL-8 increase declined 78% from 1274 $\pm$ 117 to 776 $\pm$ 41 pg/ml. This finding suggests that there is an alternative, minor pathway of CGN-induced IL-8 activation that is not mediated by TLR-4-Bcl10. *b*, similarly, following knockdown of MyD88, CGN-induced increase in Bl10, as determined by ELISA, was completely inhibited, declining from a peak of 3.83 $\pm$ 0.17 to a base line of 1.21 $\pm$ 0.08 (***, $p < 0.001$, one-way ANOVA with Tukey-Kramer post-test for multiple comparisons). *Cn*, control.

very low. The declines in fluorescence of CGN following exposure to blocking TLR4 antibody and to CGN-E are statistically significant ($p < 0.001$).

TLR4 Activation by CGN Is MyD88-dependent—When NCM460 cells were transfected with the psiRNA-hMyD88 vector to silence MyD88, CGN exposure did not increase either IL-8 or Bcl10 (Fig. 5). Vector control experiments demonstrated increases in IL-8 and Bcl10. Hence, the CGN-induced effects that are mediated by TLR4 are dependent on the adaptor protein MyD88. Differences in IL-8 and Bcl10 are statistically significant ($p < 0.001$).

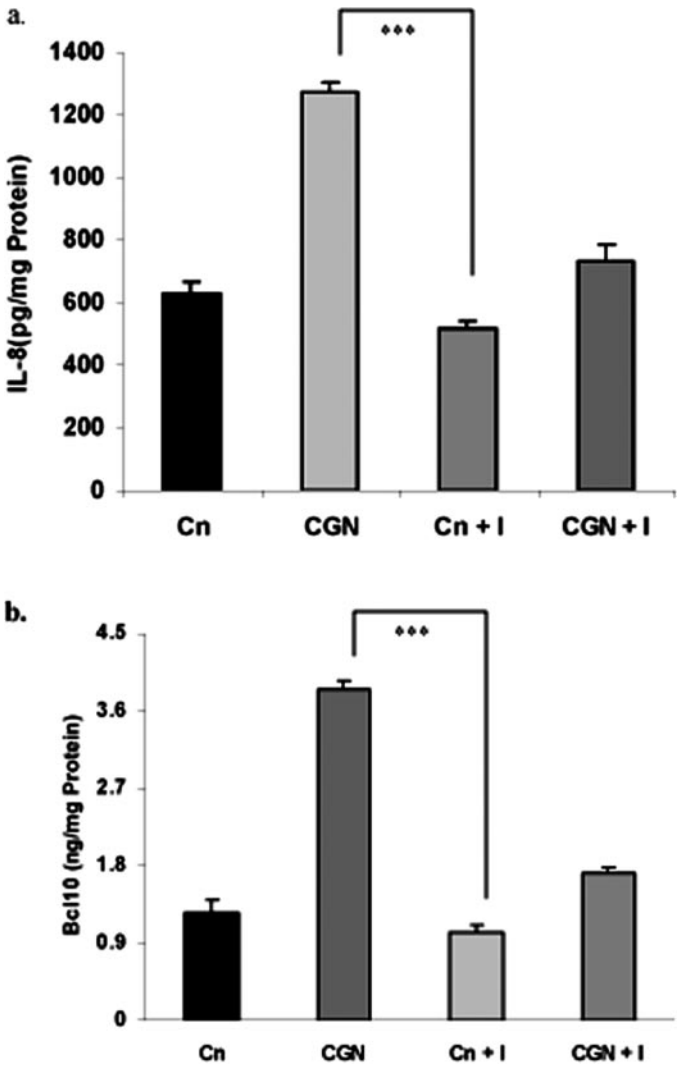

FIGURE 6. **Inhibition of IRAK reduces CGN-associated increases in Bcl10 and IL-8.** *a*, marked decline in IL-8 of the spent media follows treatment with IRAK1/4 inhibitor. *b*, similarly, a marked decline in Bcl10 protein content follows pretreatment with IRAK 1/4 inhibitor, placing Bcl10 downstream of IRAK in the cascade induced by CGN exposure. *Cn*, control; *CGN*, λCGN (1 μg/ml for 24 h); *I*, IRAK inhibitor. ***, $p < 0.001$ (one-way ANOVA with Tukey-Kramer post-test for multiple comparisons).

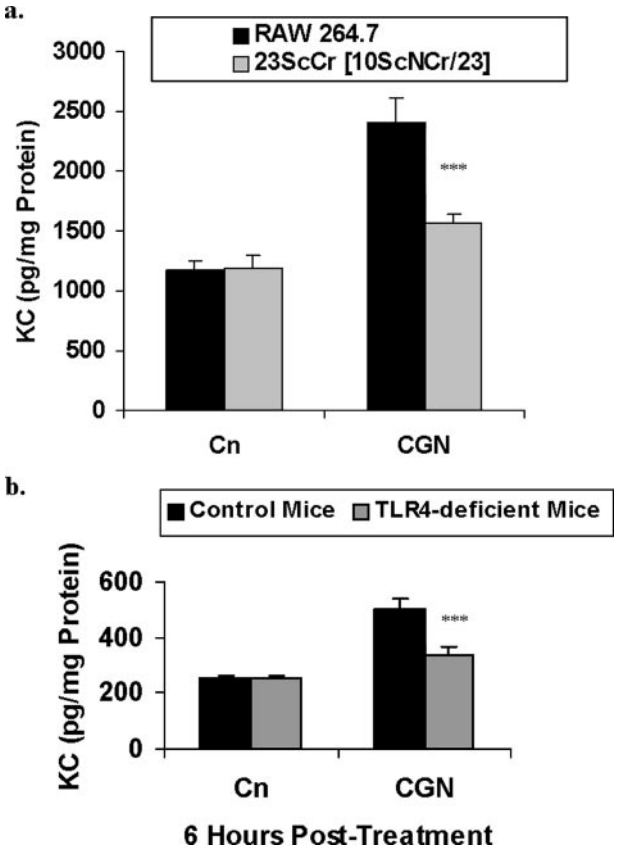

FIGURE 7. **KC secretion in TLR4-deficient mouse macrophages and colonic tissue.** *a*, KC secretion was measured by ELISA after exposure to λCGN (1 μg/ml for 24 h). The increase in KC was significantly less in the TLR4-deficient mouse macrophages (23ScCr) than in the control macrophages (RAW 264.7). ***, $p < 0.001$, one-way ANOVA with Tukey-Kramer post-test for multiple comparisons. *b*, in *ex vivo* colonic tissue from the TLR4-deficient mice (C57BL/10ScNJ), λCGN exposure (1 μg/ml for 6 h) produced significantly less increase in KC than in the normal, control mice (*Cn*) (***, $p < 0.001$, one-way ANOVA with Tukey-Kramer post-test for multiple comparisons).

Effect of IRAK1/4 Inhibitor on IL-8 and Bcl10—To ascertain the role of IRAK in the CGN-mediated cascade in NCM460 cells, the effect of exposure to an IRAK inhibitor was tested on the production of IL-8 (Fig. 6*a*) and Bcl10 (Fig. 6*b*). Both declined significantly following exposure to the IRAK inhibitor ($p < 0.001$).

KC Response to CGN in TLR4-deficient Mouse Macrophages and Mouse Colonic Tissue—KC was measured in the spent media of TLR4-deficient mouse macrophages (23ScCr) and normal macrophages (RAW 264.7). The results indicated a markedly lesser increase in KC secretion following exposure to λCGN (1 μg/ml for 24 h) in the TLR4-deficient macrophages than the controls. Base-line KC values were comparable (1194.1 ± 107.1 pg/mg protein in TLR4-deficient macrophages and 1172.7 ± 73.9 pg/mg protein in controls), rising to 1567.7 ± 65.5 pg/mg protein in TLR4-deficient mice and 2402 ± 207.2 pg/mg protein in control mice ($p < 0.001$) (Fig. 7*a*).

Similarly, following exposure of *ex vivo* colonic tissue from mice with deletion of the TLR4 gene, KC secretion post-CGN (1 μg/ml) was markedly less at 2 and 6 h as compared with the normal controls (110.6 ± 7.4 *versus* 167.4 ± 12.0 pg/mg protein at 2 h; 337.8 ± 27.7 *versus* 501.6 ± 35.6 pg/mg protein at 6 h) with similar base-line values ($p < 0.001$) (Fig. 7*b*).

Confocal Imaging of Carrageenan-treated IEC—NCM460 cells were grown in compartment slides, exposed to fluorescent-tagged λCGN for 24 h, and stained for confocal microscopy as described under "Experimental Procedures." λCGN appears *green*, nuclear DNA *blue*, and actin filaments *red* (Fig. 8). CGN has attached to a cluster of NCM460 cells at two membrane regions. Z-stack images indicated the localization of CGN at distinct sites on the surface of the group of cells.

To better localize the sites of CGN attachment, NCM460 cells were grown in Transwells for 24 h, treated with unlabeled λCGN (1 μg/ml for 24 h), and stained for TLR4 using the rabbit polyclonal antibody that recognizes the internal TLR4 epitope from residues 242–321. Cells were also stained for actin and nuclear DNA as described under "Experimental Procedures" (Fig. 9). Images of cells exposed to CGN (Fig. 9, *B* and *D*) *versus* controls (*A* and *C*) demonstrate increase in the TLR4 (*red*). Z-stack images (Fig. 9, *A* and *B*) suggest an increased presence of TLR4 toward the

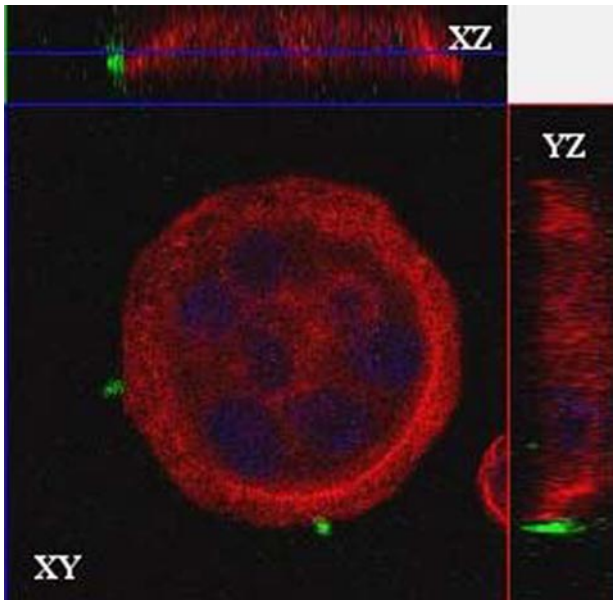

FIGURE 8. **Confocal imaging of human IEC.** Attachment of fluorescent CGN (*green*) to a cluster of human colonocytes occurred. Actin is stained *red*, and nuclear DNA is *blue*. Z-line images, seen on the *upper* and *right margins*, demonstrate the attachment of CGN-F at the lateral margins of the cell cluster. A distinct apical-basal orientation is not demonstrated.

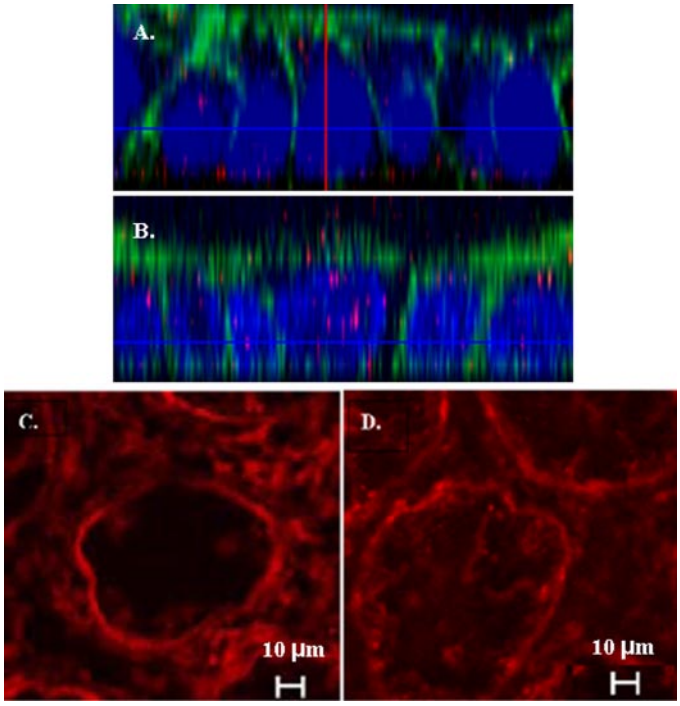

FIGURE 9. ***y-z* axis sections were obtained from NCM460 cells grown in Transwell inserts (*A–D*).** *A* and *B*, the mobility of TLR4 is demonstrated following exposure to the λCGN (1 μg/ml for 24 h). Increased staining appears at the basal membrane in the control (*A*). Increased TLR4 appears in more apical regions of the cells following CGN (1 μg/ml for 24 h) (*B*). *Yellow* staining indicates fused staining with co-localization of the TLR4 antibody (*red*) and phalloidin-stained actin (*green*). Because the epitope detected by the TLR4 antibody is an internal epitope, the extracellular domain of the TLR4 is not demonstrated. *C* and *D*, *ex vivo* human colonic sections were exposed to λCGN (1 μg/ml for 2 h) and prepared as indicated under "Experimental Procedures." TLR4 antibody from rabbit was labeled with Alex-Fluor 568 goat anti-rabbit IgG antibody. In the control (*C*), a greater concentration of the antibody along the basal aspect of the epithelial cells is evident. After exposure to λCGN for 2 h (*D*), there is more diffuse staining with the TLR4 antibody, and staining is increased more apically in the colonocytes, toward the lumen of the intestinal crypt. *Marker* = 10 μm.

apical aspect of the cells, in contrast to the basal location in the untreated control cells. *Ex vivo* sections of human colon that were exposed to λCGN for 2 h were examined following similar staining procedure. A difference in the TLR4 staining intensity along the basement membrane is evident in the treated (Fig. 9*B*) *versus* the control (Fig. 9*C*) cells. In the treated cells, the TLR4 staining is more diffuse and increases toward the apical aspect of the colonocytes and the crypt lumen.

DISCUSSION

TLR4 is an important receptor in the initiation of the inflammatory response in human cells. Found in multiple tissues, TLR4 is implicated in the innate immune response and in the recognition of pathogen-associated microbial patterns (16). Our data indicate that CGN, like LPS and lipoteichoic acid, can also activate the TLR4. Previously, we have shown stimulation of Bcl10 by CGN, consistent with activation of an innate immune response in IEC (4). Identification of TLR4 as the membrane receptor that interacts with CGN strengthens the hypothesis that the effects of CGN are attributable to activation of an innate, immune-mediated inflammatory response in human colonocytes. Because CGN is so widely used as a food additive in the Western diet, this mechanism may have implications for the development of intestinal inflammation, as well as insulin resistance, in clinical settings (20, 21, 34, 35).

The presence of CGN in food, as well as in other products such as pharmaceuticals, cosmetics, and room air deodorizers, may trigger an innate immune response, perhaps associated with the α-1,3-galactosidic epitope. Previously carbohydrates from hyaluronan have been implicated in recognition by the TLR, yet specific epitopes of the glycosaminoglycans have not been explicitly identified (36, 37).

TLR4 interaction with LPS has been associated with specific amino acids in TLR4, and single nucleotide polymorphisms or experimental modifications that alter these amino acids impede the LPS-induced inflammatory response (18, 19). The majority of the experimental work describing the impact of specific sites in TLR4 has concerned pulmonary responsiveness to endotoxin and the proclivity of some familial clusters to reduced endotoxin responsiveness. The effects of similar modifications of TLR4 on the response to CGN in the intestine have not yet been elucidated. Previously, TLR4- and MyD88-deficient mice were reported to have diminished allergic responses to λCGN (38).

The role of TLR4 in the human intestine has been investigated intensively in the past decade, although conflicting evidence has been acquired. Both increases in and reductions of TLR4 have been reported in relation to the occurrence of intestinal inflammation. It has been reported that TLR4 is absent in the epithelium in intestinal inflammation in pediatric inflammatory bowel disease (39), yet an increase in TLR4 has been identified in necrotizing enterocolitis in infants (40). In experiments with human IEC, TLR4 has been reported to be low and unresponsive to LPS (41, 42). However, other investigators have shown constitutive expression of TLR4 in T84 cells and up-regulation of TLR4 in pouchitis (43, 44). Redistribution of TLRs in IEC in association with inflammation has been observed, with transition from the apical to cytoplasmic compartments observed in polarized intestinal epithelium (43). Other reports have described perinuclear and Golgi cytoplasmic localization (23, 45). Different antibodies and antisera to TLR4

have been used in various reports, which may account for some of the observed discrepancies in cellular localization, expression, and activity (23, 33, 39–46). Our data on CGN and the TLR4 blocking antibody suggest that CGN interacts with an extracellular site on TLR4. The confocal data suggest intracellular migration of TLR4 from the basal toward the apical region following CGN exposure. The adaptor protein MyD88 appears essential for the induction of an inflammatory cascade by CGN in the IEC. Whether additional mediators, such as MD-2 and LPS-binding protein, are required is not yet known.

CGN induction of a TLR4-Bcl10-mediated pathway of intestinal inflammation is highly relevant to considerations of the pathogenicity of CGN. Bcl10 is a caspase recruitment domain (CARD) protein, as is NOD2, which has been identified as an inflammatory bowel disease gene, mutations of which are associated with Crohn disease (29, 47). This similarity suggests that both a genetic cause of inflammatory bowel disease and CGN, an environmentally linked risk factor for intestinal inflammation, may involve CARD-containing proteins. Interestingly, NOD2 has leucine-rich regions (LRR), similar to the LRR regions of TLR4. Future work may help to clarify whether there are specific interactions among the CARD and LRR domains that mediate an inflammatory cascade, as well as the precise mechanisms by which CGN interacts with TLR4.

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